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Chrysanthemum Chlorotic Mottle Viroid: a System for Reverse Genetics in the Family *Avsunviroidae* (Hammerhead Viroids)

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ABSTRACT

Viroids are small single-stranded circular RNAs able to infect plants. Chrysanthemum chlorotic mottle was one of the first viroid diseases reported, but identification and characterization of the causing RNA was delayed by its low accumulation *in vivo*. Chrysanthemum chlorotic mottle viroid (CChMVd) (398-401 nt) adopts a branched conformation instead of the rod-like secondary structure characteristic of most viroids. The natural sequence variability and the effects of artificial mutants support that the branched conformation is physiologically relevant and additionally stabilized by a kissing-loop interaction critical for RNA *in vitro* folding and *in vivo* viability. CChMVd shares structural similarities with peach latent mosaic viroid, with which forms the genus *Pelamoviroid* within the family *Avsunviroidae*. CChMVd adopts hammerhead structures that catalyze self-cleavage of the oligomeric strands of both polarities resulting from replication through a symmetric rolling-circle mechanism. The two CChMVd hammerheads display peculiarities: the plus has an extra A close to the central conserved core, and the minus an unsually long helix II. There are non-symptomatic strains (CChMVd-NS) that protect against challenge inoculation with severe strains (CChMVd-S). Introduction by site-directed mutagenesis of one of the CChMVd-NS specific mutations (UUUC→GAAA) is sufficient to change the symptomatic phenotype into non-symptomatic without altering the viroid titer. This pathogenicity determinant maps at a tetraloop of the CChMVd branched conformation. Co-inoculations with typical CChMVd-S and -NS variants showed that the infected plants remain symptomless only when the latter was in more than a 100-fold excess, indicating the higher fitness of the S variant. RNA silencing could mediate the observed cross-protection.

Keywords: Avsunviroidae, catalytic RNAs, hammerhead ribozymes, Pelamoviroid

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INTRODUCTION

Viroids are unique entities for dissecting RNA structural-functional relationships. Their minimal circular genome (246-401 nucleotides, nt) does not encode any protein, yet these RNAs contain enough information to replicate independently (without the assistance of a helper virus) in certain plant hosts, and to incite in most cases specific diseases as an indirect effect of altering the host gene expression (Diener 2003; Flores *et al*. 2005). Viroids replicate through an RNA-based rolling circle mechanism with three steps: i) synthesis of longer-than-unit strands catalyzed by a host RNA polymerase that reiteratively transcribes the initial circular template, to which the plus polarity is assigned by convention, ii) processing to unit-length mediated by a host enzyme or a viroid ribozyme, and iii) circularization resulting from the action of an RNA ligase or from self-ligation (Flores *et al*. 2005). The approximately 30 known viroids are grouped into the families *Pospiviroidae*, type species *Potato spindle tuber viroid* (PSTVd) (Diener 1972; Gross *et al*. 1978), and *Avsunviroidae*, type species *Avocado sunblotch viroid* (ASBVd) (Hutchins *et al*. 1986). PSTVd and ASBVd replicate (and accumulate) in the nucleus and the chloroplast, respectively, as most likely also do the other members of both families. This classification scheme is supported by other criteria, prominent among which is the presence in members of the family *Avsunviroidae* of hammerhead ribozymes that catalyze self-cleavage of the oligomeric strands of both polarities resulting from their rolling-circle replication. The existence of ribozymes in certain viroids support their very ancient evolutionary origin, which is independent of viruses and might go back to the RNA world postulated to have preceded the present world on Earth based on DNA and proteins.

In addition to ASBVd, the family *Avsunviroidae* is composed by *Peach latent mosaic viroid* (PLMVd) (Hernández and Flores 1992), *Chrysanthemum chlorotic mottle viroid* (CChMVd) (Navarro and Flores 1997) and *Eggplant latent viroid* (ELVd) (Fadda *et al*. 2003). Here we will focus on CChMVd, which is endowed with peculiar properties

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making it the best system for reverse genetics studies within this family.

THE DISEASE AND INITIAL SEARCH FOR A CAUSAL AGENT WITH UNUSUAL PROPERTIES

A disease termed chrysanthemum chlorotic mottle (CChM) incited by a graft-transmissible agent was first reported almost 40 years ago in commercial greenhouses of the cultivar 'Yellow Delaware' in the US (Dimock *et al*. 1971). CChM symptoms included mottling and general chlorosis of young leaves, dwarfing, and blossom delays (Dimock *et al*. 1971). However, because of the inconsistent symptom expression – infected plants may be chlorotic and 1-2 weeks later recover and then again exhibit severe symptoms (Dimock *et al*. 1971) – a systematic study was carried out. This study showed that at relatively high temperature (24-27°C) and light intensity, cuttings of the chrysanthemum cultivars 'Bonnie Jean' and 'Deep Ridge' with implants of infected tissue reacted evenly in 12-15 days first with mottling, then with sharply defined yellow and dark-green areas in the next leaves, and finally with complete chlorosis in the following leaves (Dimock *et al*. 1971; Horst 1987) (**Fig. 1**). This short reaction time, together with the easy propagation of the natural host, chrysanthemum, has very much facilitated reverse genetics approaches (see below).

Fig. 1 Typical leaf symptoms induced by CChMVd variant CM5 on chrysanthemum cv. 'Bonnie Jean' (right), compared with a healthy control of the same cultivar (left).

The agent was also readily transmitted with sap from leaves expressing the typical CChM symptoms, thus excluding a nutritional disorder and suggesting the likely involvement of a virus, a hypothesis consistent with the negative results of attempts aimed at isolating fungi and bacteria from the affected plants. However, because bioassays in diagnostic indicators discarded the participation of several known chrysanthemum viruses, and because electron microscope examinations of tissue sections and sap preparations from CChM expressing plants failed to reveal virus-like particles (Dimock *et al*. 1971), the possibility of a viroid etiology became apparent. Reinforcing this view, the CChM agent was sensitive to RNase, considerably more stable in alkaline than in acidic buffers, and it could not be sedimented by high centrifugal fields, with zonal centrifugation in sucrose gradients showing a predominant species of 6-14S (Romaine and Horst 1975). Surprisingly, attempts to identify by PAGE a CChM-associated RNA failed, not only in 5% non-denaturing gels (in which the infectivity co-migrated with the cellular 7S RNA) but also in gels of lower porosity, and assays to recover infectivity from denaturing gels were negative (Kawamoto *et al*. 1985). Although in some experiments a differential RNA was reported, it was most probably a contamination of *Chrysanthemum stunt viroid* (CSVd), a member of the family *Pospiviroidae* already identified in the same host plant. Supporting its uncommon nature, the CChM causal agent was insoluble in 2 M LiCl in contrast to other known viroids (Kawamoto *et al*. 1985). Moreover, host range studies and cross-protection bioassays (see below) also indicated that this RNA had peculiar properties.

FINDING THE ELUSIVE CAUSAL AGENT

The failure in identifying a physical entity associated to CChM raised intriguing questions, which even led to consider the possible involvement in this disease of a novel class of small pathogenic RNAs. Re-examination of where the infectivity moved in 5% non-denaturing polyacrylamide gels confirmed its location between RNA markers of 300 and 400 nt, and when the same preparations were analyzed by PAGE in 5% denaturing gels – in which circular and linear forms of viroids are considerably separated – the infectivity was recovered from the gel section around the linear RNA marker of 400 nt, whereas no symptoms were induced by the RNAs eluted from sections where viroid circular RNA markers of 250-375 nt migrate. These results indicated that the CChM causal agent was a linear RNA of approximately 400 nt, although the co-existence of a circular form accumulating at very low levels could not be dismissed (Navarro and Flores 1997). To examine the possibility that the linear RNA of approximately 400 nt causing the CChM disease could have remained masked by cellular RNAs of similar size due to the limited resolution of the short gels employed, preparations enriched in the pathogenic RNA obtained by eluting the nucleic acids from the section of a non-denaturing PAGE delimited by the linear RNA markers of 300-400 nt, were electrophoresed in a long sequencing gel under denaturing conditions. With this approach, a differential RNA was identified in infected tissue that, when purified and inoculated mechanically to chrysanthemum plants, replicated and induced the typical CChM symptoms, thus fulfilling Koch' postulates (Navarro and Flores 1997). These results, in conjunction with the subsequent identification of an accompanying circular RNA (see below) and the absence of virus-like particles in CChM-infected tissue (Dimock *et al*. 1971), showed that the causal agent was a viroid, CChMVd, and not a viroid-like satellite RNA functionally dependent on a helper virus. Moreover, *in vitro* transcripts with the complete CChMVd sequence were infectious and induced the typical symptoms of the CChM disease (Navarro and Flores 1997). Therefore, despite CChM being one of the first diseases with a presumed viroid etiology, the molecular structure of its causal agent remained an enigma for more than twenty years. The very low levels at which the linear, and particularly the circular forms of CChMVd accumulate in infected tissue, explain why previous attempts to identify this pathogenic RNA failed.

CChMVd: A CATALYTIC RNA WITH A HIGHLY BRANCHED SECONDARY STRUCTURE *IN VIVO*

Cloning CChMVd RNA presented serious difficulties as a consequence of its very low accumulation *in vivo* and the lack of any previous sequence information. To address this problem, the linear RNA inducing CChM was eluted, reverse transcribed with random hexamers, converted into a double-stranded cDNA and cloned. With this approach a partial-length (82 nt) cDNA clone was retrieved that was specific for CChMVd RNA because, when RNAs from CChM-affected plants were separated by denaturing PAGE and examined by Northern-blot hybridization with two probes of opposite polarities derived from this clone, two signals were detected with the mobility of the linear CChMVd RNAs of both polarities. Moreover, two additional signals of slower mobility and significantly less intense, which could represent the corresponding circular counterparts, were concurrently observed. To confirm that this was the case, the presumed circular CChMVd RNAs were eluted and amplified by RT-PCR using a pair of adjacent primers of opposite polarities derived from the sequence of the 82-nt fragment: a product the same length as the CChMVd linear plus RNA was obtained, which was subsequently cloned and sequenced (Navarro and Flores 1997).

The reference variant of CChMVd (CM5) turned out to be a circular RNA of 399 nt (in good agreement with the size inferred from its electrophoretic mobility in denaturing

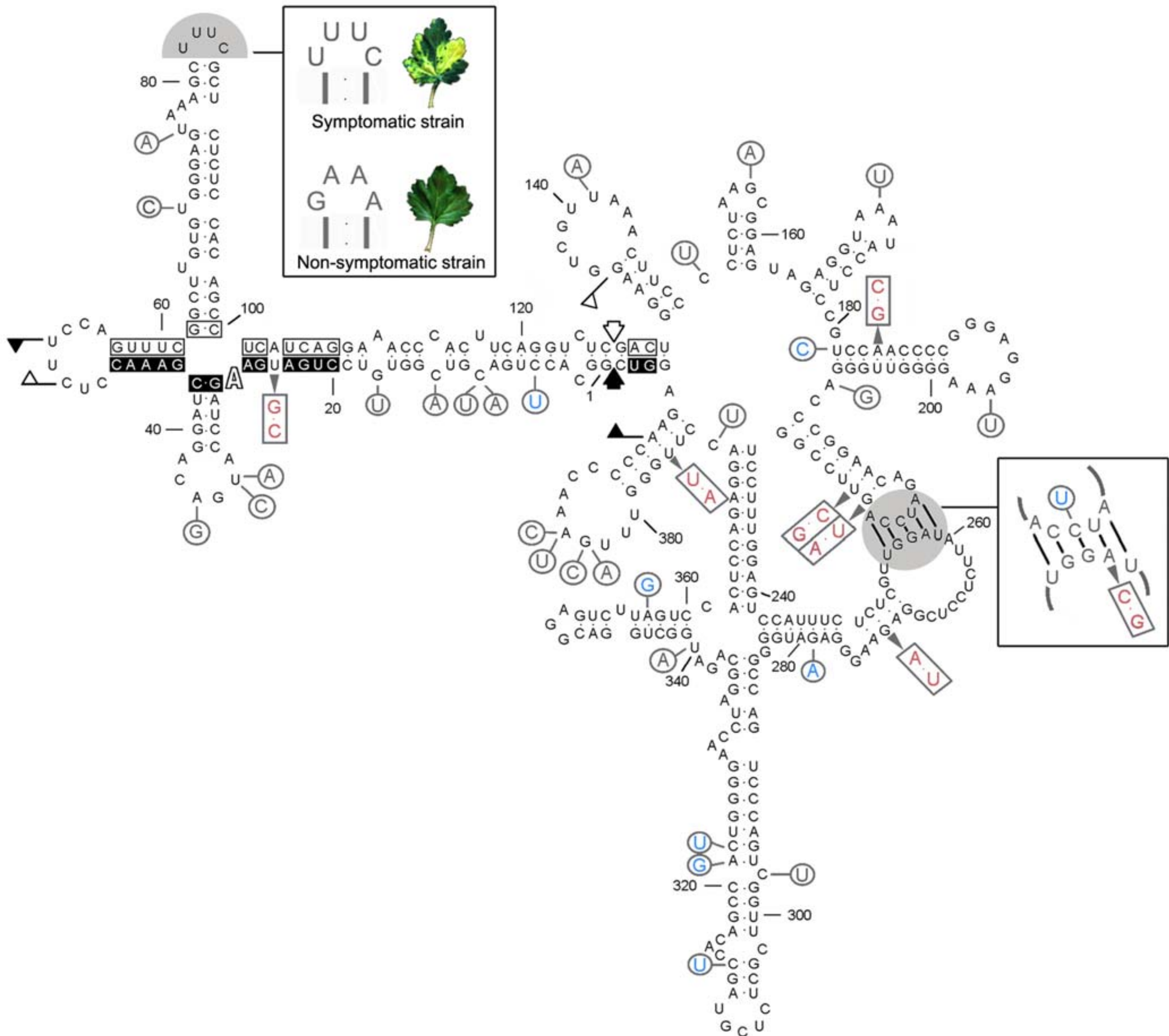

Fig. 2 Secondary structure of lowest free energy predicted for the CChMVd symptomatic variant CM5 (Navarro and Flores 1997; de la Peña *et al*. 1999). Representative sequence heterogeneities detected in other CChMVd natural variants that do not affect the secondary structure are shown in red (covariations), blue (compensatory mutations) and black (mutations in loops). Sequences forming plus and minus hammerhead structures are delimited by flags, nucleotides conserved in most natural hammerhead structures by bars, and self-cleavage sites by arrows. Solid and open symbols refer to plus and minus polarities, respectively. The outlined font indicates the position in the CChMVd secondary structure of the extra A (A10) found in its plus hammerhead structure (de la Peña and Flores 2001). The upper left gray area denotes the tetraloop at which the pathogenicity determinant has been mapped, with the two natural variants and associated symptoms being indicated in the adjacent inset). The lower right gray area denotes a kissing-loop interaction critical for *in vitro* folding and *in vivo* viability, with representative sequence heterogeneities observed in other CChMVd natural variants that support the existence *in vivo* of this element of tertiary structure being indicated in the adjacent inset (Gago *et al*. 2005).

PAGE), and a G+C content of 55.4%, also in line with the G+C content of other known viroids that, with the exception of ASBVd, is higher than 50% (Navarro and Flores 1997). Minor sequence heterogeneities were found in other variants (**Fig. 2**). Sequence analysis revealed that CChMVd did not contain the central conserved region characteristic of PSTVd and other groups of non-self-cleaving viroids but, interestingly, CChMVd did contain in both polarity strands the conserved nucleotides and the structural elements typical of the hammerhead ribozymes found in ASBVd and PLMVd, several satellite RNAs, the RNA form of a retroviroid-like element, and a transcript of the newt (Hutchins *et al*. 1986; Prody *et al*. 1986; Forster and Symons 1987; Flores *et al*. 2001). These features allocated CChMVd in the family of hammerhead viroids (*Avsunviroidae*) (Navarro and Flores 1997).

The secondary structure of lowest free energy predicted for CChMVd was a branched conformation formed by a series of stem-loops with almost 70% of the residues paired (Navarro and Flores 1997). The sequence polymorphism observed in two additional variants (CM1 and CM20) (Navarro and Flores 1997), and later on in many other variants (de la Peña *et al*. 1999; de la Peña and Flores 2002; Gago *et al*. 2005), did not affect the proposed secondary structure because the changes map at loops or because when affecting a base-pair the substitutions were covariations or compensatory mutations (**Fig. 2**). Therefore, the computer-predicted structure is most likely biologically significant, thus showing that the rod-like or quasi-rod-like secondary structure is not a universal paradigm for viroids. Moreover, this branched structure was similar to that advanced previously for PLMVd (Hernández and Flores 1992; Ambrós *et al*. 1998). In this latter viroid, *in vitro* nuclease mapping and oligonucleotide binding shift assays indicated the likely existence of a kissing-loop interaction between two hairpin loops of the branched conformation, and a comparative analysis of

PLMVd and CChMVd structures suggested the possibility of a similar interaction in CChMVd (Bussière *et al*. 2000). Structural dissection of numerous natural and artificial CChMVd variants, bioassays in chrysanthemum to assess their infectivity, and analysis of the genetic stability of the resulting progenies have proved the existence of the kissing-loop interaction in CChMVd. Furthermore, the interaction is critical for the *in vivo* viability of CChMVd and apparently it only exists in the plus polarity strand (Gago *et al*. 2005). These data indirectly support that the corresponding PLMVd interaction reported *in vitro* (Bussière *et al*. 2000) is also biologically relevant. Evolutionary conservation of a structural RNA motif usually entails conservation of biological function. Therefore, the presence of a similar kissing-loop interaction in PLMVd and CChMVd, two viroids with an overall low sequence similarity (Hernández and Flores 1992; Navarro and Flores 1997), suggests that the additional associated stability is needed in these viroids for delimiting the conformation space, avoiding kinetic traps during transcription and facilitating the adoption of a proper RNA folding. The insolubility of PLMVd and CChMVd in 2M LiCl is consistent with a folding different from the rod-like or quasi rod-like structure proposed for most viroids, which are soluble in these saline conditions (Navarro and Flores 1997). These structural similarities have led to classify CChMVd within the genus *Pelamoviroid* (Navarro and Flores 1997), the type species of which is PLMVd. From a practical perspective, CChMVd cloning and sequencing opened the way for detecting this viroid by molecular approaches that include dot-blot hybridization (de la Peña *et al*. 1999), and RT-PCR with the two pairs of specific primers used in the initial cloning and characterization (Navarro and Flores 1997; de la Peña *et al*. 1999; our unpublished results).

THE TWO UNUSUAL HAMMERHEAD RIBOZYMES OF CChMVd

Plus and minus strands of CChMVd can form hammerhead structures with the 11 conserved residues and the adjacent helices idiosyncratic of this ribozyme class. Both hammerhead structures of CChMVd have stable helices III and short loops closing helices I and II (**Fig. 3**), resembling more closely the hammerhead structures of PLMVd (Hernández and Flores 1992) than those of ASBVd (Hutchins *et al*. 1986). However, helix II of the minus CChMVd hammerhead structure is unusually long and composed of shorter helices separated by bulging residues. Moreover, when compared with other natural hammerhead structures, the plus CChMVd hammerhead structure has an extra A (A10) between the conserved A9 and the quasi-conserved G10.1 (**Fig. 3**). A10 causes a moderate decrease of the *trans*-cleaving rate constant with respect to the CChMVd plus hammerhead without this residue, whereas A10→C and A10→G substitutions have major detrimental effects, most likely because they favor catalytically inactive foldings; in contrast, A10→U substitution induces a 3-4-fold increase of the rate constant (de la Peña and Flores 2001), providing an explanation for the extra U10 present in two natural hammerheads (Flores *et al*. 2001). Because A10 also holds a singular position in a cruciform junction of the global CChMVd conformation (**Fig. 2**), this residue could have another functional role critical for its infectivity. To test this possibility, chrysanthemum plants were inoculated with recombinant plasmids containing dimeric tandem inserts of CChMVd cDNA (or with the monomeric CChMVd RNAs resulting from self-cleavage during *in vitro* transcription) with all possible mutations at this position introduced by site-directed mutagenesis. Symptoms induced by artificial variants with the substitutions A10→C, A10→U and A10→G appeared with some delay with respect to those induced by the wild-type, but none of the plants inoculated with the variant without A10 developed symptoms and dot-blot hybridization confirmed that they had not been infected. RT-PCR amplifications and sequencing of viroid progenies

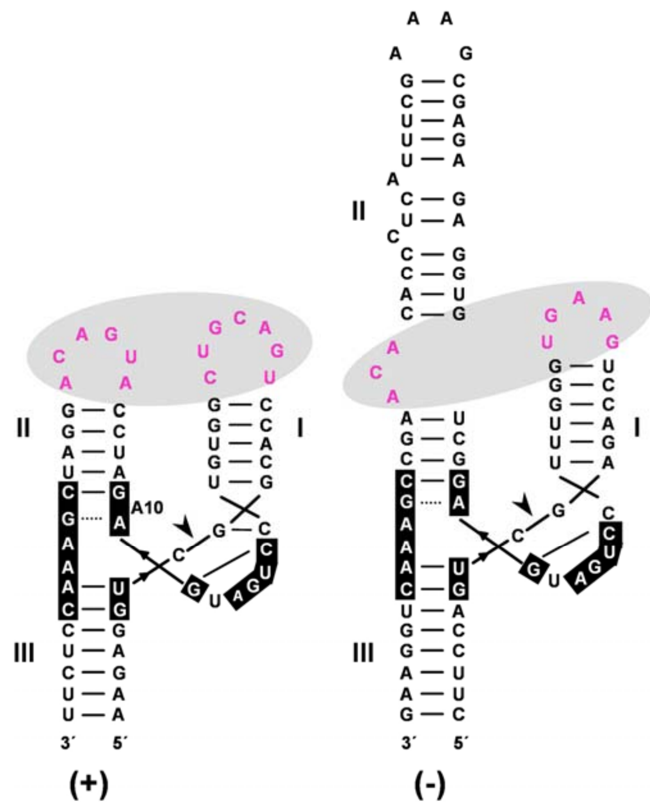

Fig. 3 Schematic representation according to X-ray crystallography of the CChMVd plus (left) and minus (right) hammerhead ribozymes of variant CM5. Nucleotides conserved in most natural hammerhead ribozymes are highlighted on a black background, and the self-cleavage sites are denoted by arrowheads. Notice the existence of an extra A (A10) in plus hammerhead structure (de la Peña and Flores 2001). Gray ovals refer to the presumed tertiary interaction between hairpin or internal loops (in magenta) enhancing the catalytic activity (Navarro and Flores 1997; de la Peña *et al*. 2003).

from the infected chrysanthemum plants revealed that the three substitutions at position 10 had reverted to the original A10 in all cases. Altogether, these results demonstrated that the A10 residue is indispensable for infectivity (its unique location should force a distortion of the cruciform domain that could be critical in interactions with other RNA regions and/or with host factors needed for CChMVd replication, transport or accumulation). Therefore, some natural hammerheads deviate from the consensus most likely due to the involvement of certain residues in critical function(s) other than self-cleavage (de la Peña and Flores 2001). Genetic information in viroids, due to their small size, is very compressed and the implication of specific regions of the molecule in more than one function can be presumed.

The efficient *in vitro* self-cleavage of plus and minus monomeric CChMVd RNAs during transcription and after purification (Navarro and Flores 1997), indicates that these reactions most probably occur through single-hammerhead structures as in PLMVd, rather than through double-hammerhead structures like those proposed in ASBVd (Flores *et al*. 2001). Two findings support an *in vivo* role for the CChMVd hammerhead ribozymes: i) the 5′ terminus of the plus linear CChMVd RNA isolated from infected tissue is identical to that generated in the *in vitro* self-cleavage reaction, and ii) the nucleotide changes detected in many CChMVd variants do not affect the stability of both hammerhead structures (Navarro and Flores 1997; de la Peña *et al*. 1999; de la Peña and Flores 2002).

The hammerhead structures of CChMVd are more closely related to each other, particularly the helix I-loop 1 domain (**Fig. 3**), than to any other hammerhead structures. This similarity, reported also between the hammerhead structures of other small catalytic RNAs (Flores *et al*. 2001),

have been suggested to result from template-switching by an RNA polymerase (Forster and Symons 1987). Recent experiments with the CChMVd plus hammerhead structure have unveiled that tertiary interactions between loops 1 and 2 in natural hammerhead ribozymes (**Fig. 3**) play an essential role in self-cleavage at the low magnesium concentrations existing *in vivo* (de la Peña *et al*. 2003; Khvorova *et al*. 2003). Due to the peculiar morphology of the CChMVd minus hammerhead structure, the tertiary interactions most likely occur between loop 1 and an internal loop of helix II (**Fig. 3**). As already indicated, CChMVd hammerhead structures catalyze self-cleavage of the oligomeric strands of both polarities resulting from replication through a rolling-circle mechanism. Details about the preceding and succeeding steps of the replication cycle are essentially absent but, presumably, they resemble those of PLMVd (Flores *et al*. 2006).

DISSECTING THE PATHOGENICITY DETERMINANT OF CChMVd

Early biological studies (Horst 1975) postulated the existence of an infectious but non-symptomatic (NS) strain of CChMVd, not yet identified as a viroid at that time, to explain why some chrysanthemum plants of a sensitive cultivar did not develop the characteristic symptoms when inoculated with a severe (S) strain of CChMVd. This observation was assumed to result from a cross-protection phenolmenon – plants infected with a NS strain would be protected against the challenge inoculation by a S strain of the same or very similar agent (see below) – and, consistent with this view, cross-protection bioassays revealed the existence of a transmissible agent that protected against CChM disease (Horst 1975). However, definitive physical evidence supporting this contention had to wait for more than 20 years until these experiments were reproduced, and Northern-blot hybridizations provided direct proof for the existence of a CChMVd-NS RNA in healthy-looking plants of a sensitive chrysanthemum cultivar. Moreover, the intensity of the hybridization signals generated by CChMVd-S and -NS RNAs was similar, indicating that their associated phenotypes were not the consequence of different accumulation levels in the infected tissue (de la Peña *et al*. 1999). These results paved the way for the molecular characterization of the pathogenicity determinant of CChMVd.

Analysis of CChMVd-NS cDNA clones, obtained by RT-PCR, revealed a size and sequence very similar to those of the CChMVd-S strain. Some of the mutations observed in CChMVd-NS variants were previously identified in CChMVd-S, but others were new. When bioassayed in chrysanthemum, cDNA clones containing the CChMVd-NS specific mutations were infectious, but non-symptomatic. Site-directed mutagenesis showed that one of the CChMVd-NS specific mutations, a UUUC to GAAA substitution, was sufficient to change the symptomatic phenotype into non-symptomatic without altering the final accumulation level of the viroid RNA. The pathogenicity determinant, the first of this class identified in a member of the family *Avsunviroidae*, mapped at a tetraloop of the computer-predicted branched conformation for CChMVd RNA. Furthermore, the sequence heterogeneity observed in variants from both strains strongly supported the existence of such a conformation *in vivo* (de la Peña *et al*. 1999).

Additional molecular dissection of this tetraloop was performed by site-directed mutagenesis, bioassay of the CChMV-cDNA clones and analysis of the resulting progenies. None of the changes introduced in the tetraloop, including its substitution by a triloop or a pentaloop, abolished infectivity. Moreover, the thermodynamically stable GAAA tetraloop characteristic of CChMVd-NS strains was not functionally interchangeable for other stable tetraloops of the UNCG family, suggesting that the sequence, rather than the structure, is the major factor governing conservation of this motif. In most cases the changes introduced initially led to symptomless infections, which eventually evolved to symptomatic concurrently with the prevalence in the progeny of the UUUC tetraloop characteristic of CChMVd-S strains. Only in one case the GAAA tetraloop emerged and eventually dominated the progeny in infected plants that were non-symptomatic. These results revealed two major fitness peaks in the tetraloop (UUUC and GAAA), with the adjacent stem being also under strong selection pressure (de la Peña and Flores 2002).

CChMVd: CROSS-PROTECTION AND RNA SILENCING

CChMVd has a very restricted host range. Of 51 species belonging to 35 genera tested, including nine chrysanthemum species and cultivars thereof, CChMVd only infected two species of chrysanthemum, *Chrysanthemum moriflorum*, now *Dendranthema grandiflora*, and *C. zawadskii*, but not 11 species of plants known to support replication of other viroids (Horst 1987). The host range of ASBVd and ELVd is also essentially limited to their natural hosts (Desjardins 1987; Fadda *et al*. 2003), whereas there is a discrepancy on whether PLMVd is restricted to peach (and peach hybrids) or infects a wide assortment of *Prunus* and non-*Prunus* species (Flores *et al*. 2007). Therefore, host-specificity may well be a common trend for members of the family *Avsunviroidae*, perhaps related to their chloroplast localization, which could involve specific mechanisms for access to and replication within this organelle. Transmission of CChMVd by insects or pollen has not been reported. However, considering that at least another member of its family (ASBVd) is pollen-transmitted and that many chrysanthemum cultivars produce high amounts of pollen, this question deserves careful examination. Control measures are restricted to the use of viroid-free propagating material, to the regular decontamination of pruning implements with diluted comercial bleach, and to early detection of the viroid and subsequent removal of the infected plants.

As already mentioned, early reports (Kawamoto *et al*. 1985) that were confirmed once the CChM causal agent was identified as a viroid (Navarro and Flores 1997), revealed singular physical properties of this RNA illustrated by its insolubility in 2 M LiCl in contrast to most viroids. In support of the peculiar nature of CChMVd, co-inoculation experiments showed that CSVd, *Citrus exocortis viroid* (CEVd), and a mild and a severe strain of PSTVd, but not the agent of CChM disease, exhibited cross-protection in a variety of combinations, suggesting that the latter was different from the other three known viroids (Niblett *et al*. 1978). Cross-protection bioassays also advanced the existence of NS strains of the CChM causal agent, and eventually lead to its identification and to the characterization of the correspondding pathogenicity determinant (see above). Moreover, co-inoculations with defined CChMVd-S and -NS variants showed that only when the latter was in a 100- to 1000-fold excess did the infected plants remained symptomless, confirming the higher biological fitness of the S variant and explaining the lack of symptom expression previously observed in cross-protection experiments (de la Peña *et al*. 2002).

Because viroids lack any protein-coding capacity, the cross-protection effects observed in CChMVd and other viroids must be necessarily RNA-mediated. This particular class of RNA-mediated cross-protection is functionally equivalent to post-transcriptional gene silencing (PTGS) (Ratcliff *et al*. 1999), an RNA-based regulatory layer discovered recently in eukaryots with many ramifications including defense against foreign pathogens (Baulcombe 2004). In this latter instance, the characteristic markers of PTGS are the small interfering RNAs (siRNAs) resulting from the action of a host RNase III-like enzyme – Dicer, or Dicer-like (DCL) in plants – on double- or quasi-double-stranded RNAs of the invading pathogen. The finding of viroid-specific siRNA of both polarities in tissues infected by representative members of both viroid families including CChMVd (Martínez de Alba *et al*. 2002; Flores *et al*. 2005),

indicates that they are targets of one or more DCL isoenzymes. CChMVd- and PLMVd-specific siRNAs are particularly abundant in infected leaves, in contrast to their corresponding genomic RNAs that accumulate to very low levels (Martínez de Alba *et al*. 2002). Because the opposite situation occurs in ASBVd-infected tissue, this inverse correlation suggests a feed-back regulatory circuit between the genomic RNA and the viroid-specific siRNAs (at least in the family *Avsunviroidae*) (Martínez de Alba *et al*. 2002). These latter RNAs would then load the RNA induced silencing complex (RISC), another component of the RNA silencing pathway that contains an RNase with specificity for single-stranded RNAs, and direct this complex for degrading their corresponding viroid genomic RNAs. In this framework, the cross-protection observed in CChMVd can be interpreted by assuming that the siRNAs generated by the pre-inoculated NS strain load and guide RISC against the genomic RNA of the challenging S strain.

CONCLUSIONS AND PERSPECTIVES

The system formed by CChMVd and chrysanthemum has unique advantages for the study of viroid-host interactions within the family *Avsunviroidae*. The natural host is also a convenient experimental host because it is easy to propagate vegetatively and to inoculate mechanically with CChMVd-specific DNA or RNA preparations, and it expresses characteristic symptoms in a relatively short time. These advantages have facilitated along the recent years significant progress in different areas, particularly in those involving reverse genetics approaches. The system, however, is inappropriate for other studies that demand high accumulation levels of the genomic RNA in infected tissues; for such purposes, the system ASBVd-avocado is most likely the best option.

What else can be learned using CChMVd and its natural host? Because chrysanthemum displays a strong RNA silencing response against CChMVd, as revealed by the abundant viroid-specific siRNAs in infected tissues (Martínez de Alba *et al*. 2002), this system appears specially suitable for RNA silencing studies including molecular characterization of the siRNAs from S and NS strains and their subcellular location, which ultimately may provide deeper insights into the mechanisms underlying cross-protection and pathogenesis. Furthermore, because chrysanthemum is also a host for CSVd, a member of the family *Pospiviroidae*, the response of a common host to two very different viroids can be studied as a recent report illustrates (Codoñer *et al*. 2006). In the context of hammerhead ribozymes, dissection of the atypical CChMVd minus hammerhead structure should confirm and define the tertiary interactions predicted between loop 1 and an internal loop of helix II. Last but not least, the system CChMVd-chrysanthemum may be instrumental for understanding how an RNA moves through the chloroplastic membrane, a question that goes beyond the limits of virology because no endogenous or foreign RNAs (excepting members of the family *Avsunviroidae*) have been reported to cross this barrier.

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